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RESEARCH

Characterization of outer membrane vesicles from *Acinetobacter haemolyticus* and their role in the dissemination of carbapenem resistance

Elsy Martínez-Gorgonio[1] · Rosa del Carmen Rocha-Gracia[1] · Miguel Castañeda-Lucio[1] · Margarita M. P. Arenas-Hernández[1] · Miguel A. Cevallos[2] · Yolanda Sáenz[3] · Patricia Lozano-Zarain[1]

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Abstract

Species outside the *Acinetobacter calcoaceticus–baumannii* (Acb) complex have recently emerged as new clinically relevant carbapenem-resistant pathogens, such as *A. haemolyticus*. A new mechanism that could contribute to the dissemination of this resistance is the secretion of outer membrane vesicles (OMVs). We investigate OMVs secreted by *A. haemolyticus* AN54, a Mexican clinical strain carrying a plasmid-borne bla_{NDM-1} gene and by its plasmid-cured derivative AN54Δe. Active secretion of OMVs formed by a lipid membrane, measuring 10–50 nm in both strains, was observed by TEM. The OMVs from AN54 possess hydrolytic capacity against carbapenem. The OMV protein profile varied depending on the antibiotic concentration during induction, with additional protein bands detected at 8 and 32 µg/ml of antibiotic exposure. Mass spectrometry identified proteins involved in different metabolic pathways and in resistance-related processes. The porins, efflux pumps, and ADC enzyme were more abundant in the OMVs from AN54Δe than in those from AN54 harboring the active NDM-1 enzyme. The OMVs protected the sensitive strains from the same and different genera against the antibiotic action for a limited period. However, the OMVs transferred the bla_{NDM-1}-carrying plasmid (pAhaeAN54e) only to the AN54Δe strain, conferring permanent resistance to carbapenems. Together, these findings suggest that OMVs can help protect surrounding bacteria from antibiotic action and serve as vehicles for disseminating resistance genes. These results show the importance of studying OMVs as a novel mechanism for the dissemination of antimicrobial resistance.

Keywords Acinetobacter · OMVs · NDM-1 · Proteomics · Dissemination of resistance

✉ Patricia Lozano-Zarain
patricia.lozano@correo.buap.mx;
plozano_zarain@hotmail.com

Elsy Martínez-Gorgonio
elsy.martinezgo@alumno.buap.mx

Rosa del Carmen Rocha-Gracia
rosa.rocha@correo.buap.mx

Miguel Castañeda-Lucio
miguel.castaneda@correo.buap.mx

Margarita M. P. Arenas-Hernández
margarita.arenas@correo.buap.mx

Miguel A. Cevallos
mac@ccg.unam.mx

Yolanda Sáenz
ysaenz@riojasalud.es

[1] Posgrado en Microbiología, Centro de Investigaciones de Ciencias Microbiológicas, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, Edificio IC-11, Ciudad Universitaria, Colonia Jardines de San Manuel, CP. 72570 Puebla, PUE, Mexico

[2] Programa de Genómica Evolutiva, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

[3] Área de Microbiología Molecular, Centro de Investigación Biomédica de La Rioja (CIBIR), Logroño, Spain

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Introduction

The acquisition and dissemination of resistance genes in bacteria are strongly associated with mobile genetic elements that facilitate horizontal gene transfer, with plasmids playing a key role in this process. Although the most studied transfer mechanisms are transformation, transduction, and conjugation, new forms have been discovered in the last decade, such as outer membrane vesicles (OMVs), that contribute to the dissemination not only of antibiotic resistance genes but also of functional enzymes as beta-lactamases [1–3].

In bacteria, extracellular vesicles are named based on the characteristics of the microorganism that secretes them. They are known as cytoplasmic membrane vesicles (CMVs) in gram-positives, while they are OMVs in gram-negatives. OMVs are nanostructures mainly composed of DNA, RNA, LPS, phospholipids, outer membrane proteins, and periplasmic proteins. Different studies have been carried out using transmission electron microscopy in *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Neisseria gonorrhea*, observing OMVs with an estimated diameter ranging from 20 to 400 nm [4–9].

In the last 50 years, the study of these OMVs has revealed their involvement in various biological processes, such as toxin and bacteriocin release and waste material excretion, intra- and extracellular communication, and immunomodulation [4, 10, 11]. Examples include Shiga-toxin and hemolysin realized by some *E. coli* pathotypes or the packaging of hydrolases, phospholipase C, alkaline phosphatase, protease, elastase, hemolysins [12–14]. Additionally, it has been reported that they may contribute to the dissemination of small DNA fragments carrying resistance genes to bacterial communities to combat the action of antibiotics. This phenomenon has been observed in hypervirulent *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, showing the transfer of resistance among bacteria from the same or different genera [2, 15–17].

Recent studies have reported the release of resistance genes and proteins, such as beta-lactamases, through OMVs [8, 18–20]. Other authors have reported the carbapenem-hydrolytic capacity mediated by OMVs in imipenem-induced KPC-carrying *K. pneumoniae* [20]. On the other hand, in *A. baumannii* OMVs, proteins with hydrolytic potential, such as Metallo-beta-lactamase NDM-1, and some resistance genes such as $bla_{\text{OXA–24}}$, $bla_{\text{NDM–1}}$, $bla_{\text{OXA–51}}$, $bla_{\text{OXA–133}}$, and *aac(6')-Ib-cr* have been identified as part of their luminal cargo. These genes can be transferred to the same genus and different genera, like *E. coli* [2, 16, 21, 22]. The protein cargo of *A. baumannii* OMVs is highly complex, as it includes serine proteases implicated in pathogenesis, porins, and membrane proteins, such as OmpA (involved in cytotoxicity, adherence and invasion), OmpW (involved in immune response activation), CarO, and OprD, participating in carbapenem and beta-lactam resistance, respectively, in addition to other proteins that play important roles in bacterial virulence [23, 24]. These findings have significant clinical implications as *A. baumannii* is considered a main resistance gene reservoir and a prevalent causative agent in healthcare-associated infections (HAIs) in recent decades; it has even been considered an endemic and responsible bacterium of outbreaks in numerous hospitals [25]. The limited number of studies on non-*baumannii* species has an impact on the understanding of OMVs role in other *Acinetobacter* species, such as *haemolyticus*, *lwoffii*, *junii*, *soli*, *ursingii*, *bereziniae*, *johnsonii*, *radioresistens*, and *parvus*, whose relevance has increased due to their high frequency of resistance gene acquisition [3, 26–28].

Acinetobacter spp. are considered important carriers of carbapenem-resistance genes, such as $bla_{\text{NDM–1}}$ and some bla_{OXA} variants, which could influence the biogenesis and secretion of OMVs. NDM-1 is a carbapenemase anchored to the outer membrane of gram-negative bacteria due to a lipidation amino acid sequence at one end of the signal peptide, which distinguishes it from other soluble metallo-β-lactamases found in the periplasm. This anchoring not only improves the enzyme stability but also facilitates its release through OMVs [18, 29–31]. The carriage of serine beta-lactamases, metallo-beta-lactamases, and even their genes inside OMVs has already been reported in several bacterial models, indicating that it is a common event. Hence, its investigation is important to understand what role they may be playing in current antibiotic resistance [2, 7, 16, 32, 33]. In this context, the characterization of OMVs from a clinical strain outside the Acb complex, such as *A. haemolyticus*, which harbors five plasmids, one of which carries NDM-1, could provide crucial information about the dissemination and spread of antimicrobial resistance. This study highlights the role of OMVs as carriers of active NDM-1 and protectors through antibiotic degradation and their potential contribution to antibiotic degradation in a polymicrobial consortium, which could lead to serious implications for public health.

Materials and methods

Bacterial strains

The strain *Acinetobacter haemolyticus* AN54, previously studied in our group, was used; this strain was isolated from a patient admitted to a hospital in Puebla, Mexico, has chromosome of 3,600,228 bp and five plasmids: plUnnamed

(6,404 bp), pAhaeAN54c (11,418 bp), pAhaeAN54d (12,840 bp), pAhaemAN54a (4,723 bp), and pAhaeAN54e (45,460 bp). pAhaeAN54e carry resistance genes, where bla_{NDM-1} and *aphA6* were found (GenBank CP041224.1) [26]. The *A. haemolyticus* AN54 that lost the plasmid carrying the bla_{NDM-1} was used as a control (AN54Δe).

OMV obtaining conditions

The strains AN54 and AN54Δe were used to establish the conditions for the secretion of OMVs. The strains were grown in Brain Heart Infusion (BHI) and Luria Bertani (LB) broth and agar at 37 °C and 40 °C with agitation (150 rpm) and different incubation times of 16, 24, 48, 72, and 96 h. After each condition, a culture aliquot was taken, and a Gram stain was performed to evaluate bacterial and OMVs morphology.

Growth conditions and induction of OMV secretion

Both strains were grown in BHI broth (BD Bioxon) at 37 °C with agitation (150 rpm) for 16 h; in addition, OMV secretion in AN54 was induced by supplementing the growth medium with different concentrations of imipenem and meropenem (8-, 32-, and 128-µg/ml).

Obtaining and purifying OMVs

Isolation of OMVs was performed as described by Chatterjee et al. [2] with some modifications. Bacteria were cultured in BHI (BD Bioxon®) at 37 °C for 16 h in agitation. The supernatant of the culture was filtered through membranes with pore sizes of 0.65-, 0.45-, and 0.22-µm (Millipore™, 25 mm). Subsequently, cold acetone (BAKER ANALYZED® ACS, J.T.Baker®) was added to the supernatant at a 1:1 ratio and maintained at 4 °C overnight. OMVs were then recovered by centrifugation at 6400 g for 25 min at 4 °C. The resulting pellets were resuspended in PBS 1X pH 7.4 and stored in Eppendorf tubes at 4 °C until use (maximum 15 days). The sterility of the OMVs was assessed by plating 2 µl of the OMVs on TSA plates. The concentration of purified OMVs was indirectly determined by protein concentration using the Bradford assay (BioRad Laboratories).

Transmission electron microscopy

Transmission electron microscopy (TEM) of the strains AN54 and AN54Δe secreting OMVs was performed as described by Dhurve et al. [22], with minor modifications. Briefly, 10 µl of a culture of AN54 grown for 16 h in BHI medium with imipenem (8 µg/ml) was fixed onto a copper grid (200 rpm) for 20 s; the excess liquid was removed using filter paper. Subsequently, flotation staining was performed with 1% uranyl for 60 s, and the excess stain was removed with filter paper by gently touching the grid. Finally, the dried grid was visualized under a transmission electron microscope (JEM 1400, JEOL LTD, Japan) in the Electron Microscopy Unit (UME), LaNSE, CINVESTAV-Instituto Politécnico Nacional.

Determination of carbapenemase activity in OMVs

The carbapenemase activity was performed by the carbapenem inactivation test described by Yao et al. [34], with some modifications. The test is based on the observation that a decrease in the inhibition zone diameter of the sensidisc on the susceptible strain indicates carbapenem hydrolysis by the bacteria or OMVs. Different tubes containing different samples: 1.5×10^8 UFC/ml bacterial suspension of AN54 (positive control), 0.120 µg/µl of protein of OMVs from AN54Δe (negative control), and different samples containing OMV from strain AN54 induced with different carbapenems concentrations (8-, 32-, and 128- µg/ml of IMI and MEM). Subsequently, a disc of IPM (Imipenem 10 µg) or MEM (Meropenem 10 µg) Sensi-Disc (BBL™ Sensi-Disc™) was added to each sample tube and incubated for 4 h at 37 °C with agitation (150 rpm). The discs were placed on a plate with carbapenem-sensitive *E. coli* ATCC 25,922 lawns. The test was considered positive if there was a decrease in the diameter of the inhibition halo compared with the negative control halo. The test was performed in triplicate in three independent experiments. Data were analyzed using GraphPad Prism 9 (GraphPad Software, CA, USA). Statistical significance was considered if the *p*-value was < 0.0001.

SDS-PAGE analysis of bacterial proteins and OMVs

Proteins were separated using SDS-PAGE gel to obtain the protein profiles of both bacteria and OMVs. We loaded 18 µg of protein from each sample (bacterial and OMVs) with 30 µl of loading buffer (Tris 65 mM, SDS 4%, β-mercaptoethanol 10%, glycerol 20%), which were boiled at 100 °C for 10 min. The samples were separated on a 10% SDS-PAGE gel and later stained with Coomassie Brilliant Blue G-250 (Bio-Rad) at 1% [22]. Each assay was performed in triplicate using independently purified OMVs.

Identification of OMVs proteins by mass spectrometry

Highly complex mass spectrometry-based proteomics analysis (the most sensitive assay performed with the available equipment), was performed in the Genomics, Proteomics

and Metabolomics Core Facility (UGPM), LaNSE, CINVESTAV-Instituto Politécnico Nacional. The analysis was performed against the *Acinetobacter haemolyticus* AN54 database (GenBank CP041224.1).

Bioinformatic analysis

The proteins obtained by function and classification were analyzed on the STRING platform [35]: https://string-db.org/cgi/input?sessionId=bsbOgFK1i96X. The results were analyzed using a web-based tool that analyzes sets through Venn diagrams [36] (https://www.interactivenn.net/).

Protection assay of OMVs in strains of different genera susceptible to imipenem

The protection conferred by OMVs was evaluated following the protocol described by Zhang et al. [20] with some modifications. OMVs from AN54, obtained with and without induction using different imipenem and meropenem concentrations of 8-, 32-, and 128 µg/ml, were used. In a 96-well plate, 50 µl of a suspension of the susceptible strains adjusted to 0.5 on the McFarland scale was co-incubated with 0.5 µg/µl and 1.5 µg/µl of protein of the OMVs for 30 min; subsequently, imipenem (8 µg/ml) was added. The plates were then incubated at 37 °C for 24 h. The contents of each well were then subcultured onto BHI agar without antibiotics; after 24 h of incubation at 37 °C, two additional subcultures were performed on BHI agar supplemented with 8 µg/ml of imipenem. The susceptible strains were *A. baumannii* AE12, *E. coli* ATCC25922, and *A. haemolyticus* AN54Δe. Protection was evidenced by the growth of the susceptible strain in the BHI plate in the presence of imipenem at 8 µg/ml. The OMVs from AN54Δe were used as a negative control. The assay was performed in triplicate, obtaining the OMVs purified for each replicates.

Characterization of transformants strains

During the protection assay, we used purified OMVs (carriers of the pAhaeAN54e plasmid) obtained from AN54, which were previously cultured on BHI agar to ensure their sterility. Once confirmed, the OMVs were co-incubated with pure cultures of sensitive bacteria such as *A. baumannii* AE12, *E. coli* ATCC25922, and *A. haemolyticus* AN54Δe (plasmid pAhaeAN54e-cured strain). Strains were defined as transformants when they were able to grow for at least three consecutive passages on antibiotic-containing agar plates (8 µg/µl of IMI), from which they were subsequently recovered for further analysis. The acquisition of resistance to carbapenems by the transformed strains was confirmed through various assays: their activity was determined by the CarbAcineto NP test described by Dortet et al. [37], this assay is based on the pH-dependent colorimetric detection of imipenem β-lactam ring hydrolysis. The results of this test were interpreted one hour after incubation at 37 °C. A change in phenol red from red to yellow was considered a positive result. Also, the presence of the bla_{NDM-1} was confirmed by PCR using primers NDMprotF: CTCGAGATGG AATTGCCCAATATTAT and NDMprotR: GGTACCGCG CAGCTTGTCGGCCAT. The strain *A. haemolyticus* AN54 was used as a positive control in both assays.

On the other hand, the S1 nuclease-pulsed-field gel electrophoresis (PFGE) method was carried out to determine the presence of the resistance plasmid, using *Escherichia coli* NCTC 50,192 and *A. haemolyticus* AN54 strains as a reference [26].

Results

Obtention of OMV from *Acinetobacter haemolyticus* AN54 and AN54Δe

Different culture media and incubation times were evaluated to determine the optimal conditions for producing OMVs (as described in Materials and Methods). We observed that bacterial growth was higher in BHI than in LB. OMVs from all conditions were observed by Gram staining to obtain a preliminary result about their characteristic (Fig. S1, Supplementary). We observed OMVs as small spherical structures, whose apparent size was smaller than that of a bacterium. In addition, the number of OMVs increased proportionally with the incubation time, which enhanced the likelihood of obtaining type E OMVs and, also showed greater self-aggregation after 24 h. Based on these experiments, we decided to use BHI broth and incubate at 37 °C for 16 h with shaking (150 rpm) to obtain OMVs from AN54 and AN54Δe, to continue with the study.

Transmission electron microscopy characterization of OMVs

To identify the morphological characteristics of OMVs, both free and in the process of secretion, transmission electron microscopy (TEM) was used on the AN54 strain induced with imipenem. The purified OMVs of AN54 obtained after induction with 8 µg/ml of imipenem for 16 h were analyzed by TEM to observe their characteristics; however, the purified OMVs were observed as self-aggregated particles, which hindered their proper visualization (data not shown). Therefore, we decided that TEM was performed during the active secretion of OMVs. The induction was performed using a concentration of 8 µg/ml of imipenem

to stimulate OMV secretion and allow a more detailed observation of the individual characteristics of the control strain AN54Δe (Fig. 1a) and the strain AN54 (Fig. 1b–d), subjected to induction with 8 µg/ml of imipenem for 16 h. OMV release from AN54 (Fig. 1b–d) was observed on the cell surface as small spherical structures in intact and dividing cells. A single membrane corresponding to OMV type B was identified. The OMV population was homogeneous, with a size range from 10 to 50 nm. When comparing the OMVs secreted by AN54 and AN54Δe, a slight variation in size was observed, with OMVs from AN54Δe being slightly larger; however, this difference was not significant. In terms of structure, both exhibited similar characteristics.

Determination of the carbapenemase activity present in the OMVs

Considering that AN54 carries NDM-1, a membrane-bound metallo-β-lactamase, we evaluated whether OMVs contained NDM in their membrane and exhibited activity against imipenem and meropenem. Using the carbapenem inhibition technique, we observed that the activity in the OMVs obtained with induction with 8 µg/ml of imipenem shows a slight degradation of imipenem but not of meropenem; nevertheless, the OMVs obtained with induction with 32 and 128 µg/ml show an absolute degradation of imipenem and meropenem (Fig. 2a).

In contrast, OMVs obtained after induction with meropenem at 8 µg/ml showed that they degraded imipenem slightly, but not meropenem. The OMVs obtained with induction with 32 and 128 µg/ml of meropenem showed an absolute degradation of imipenem; however, no significant changes were observed in the hydrolysis of meropenem (Fig. 2b).

Analysis of the protein cargo of OMVs secreted by *A. haemolyticus* AN54

We analyzed the protein profile of OMVs obtained by induction with imipenem and meropenem at different concentrations through SDS-PAGE. Protein profile analyses were performed in triplicate using OMVs isolated from three independent experiments. The OMVs were obtained under different induction concentrations with imipenem and meropenem. All these allow for the evaluation of the reproducibility of the observed protein pattern. We can observe that the protein profiles of the bacterial *A. haemolyticus* AN54 without induction and AN54Δe (Fig. S2a and b, lanes 2 and 3 Supplementary, respectively) are very similar, even though only AN54 carries NDM-1.

In contrast, the protein profiles of the OMVs purified and obtained without induction from AN54 (Fig. S2a and b, lane 5 Supplementary) show a protein profile different from that observed in the non-induced OMVs from AN54Δe (Fig. S2a and b, lanes 6 Supplementary). On the other hand, comparing the protein profile from OMVs obtained without induction with those induced with different concentrations of imipenem (Fig. S2a, lanes 7 to 9 Supplementary), we can observe differences in the protein profile.

When comparing the protein profile of OMVs induced with imipenem, an increase in protein banding was observed, especially at concentrations of 8- and 32 µg/ml. In the case of OMVs induced with meropenem, the greatest banding was recorded at 32 µg/ml. In contrast, non-induced

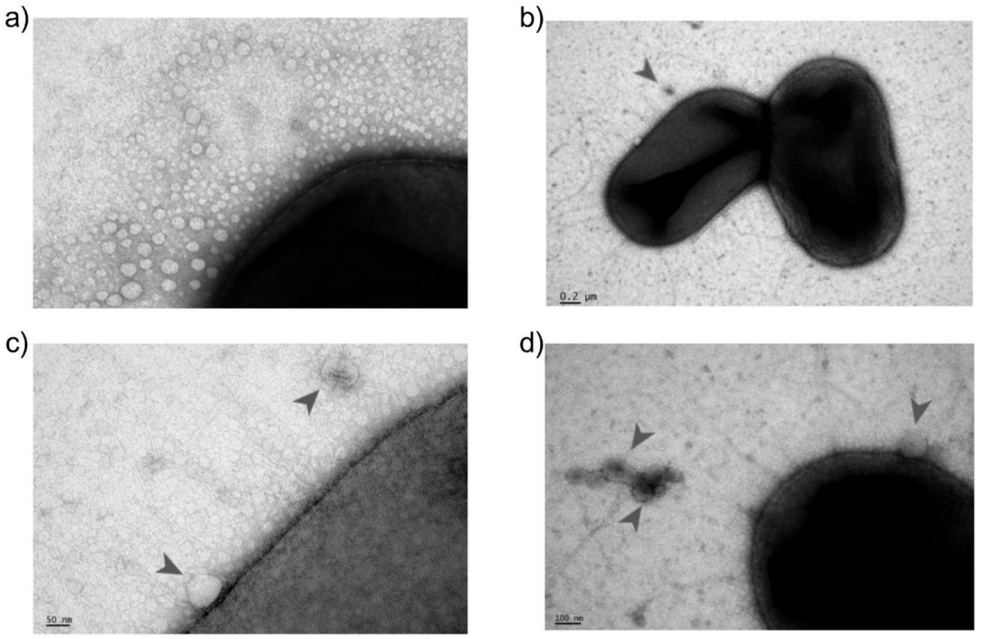

Fig. 1 Transmission electron micrographs of OMV secretion after induction with 8 µg/ml of imipenem in the logarithmic phase (16 h, 37 °C, BHI medium) **a** AN54Δe control strain, **b***Acinetobacter haemolyticus* AN54, **c** High-magnification microscopy of the OMVs in figure b (magnification of ×100,000), **d** Secretion and membrane fusion events involving multiple OMVs of AN54. The black arrows indicate some of the released OMVs

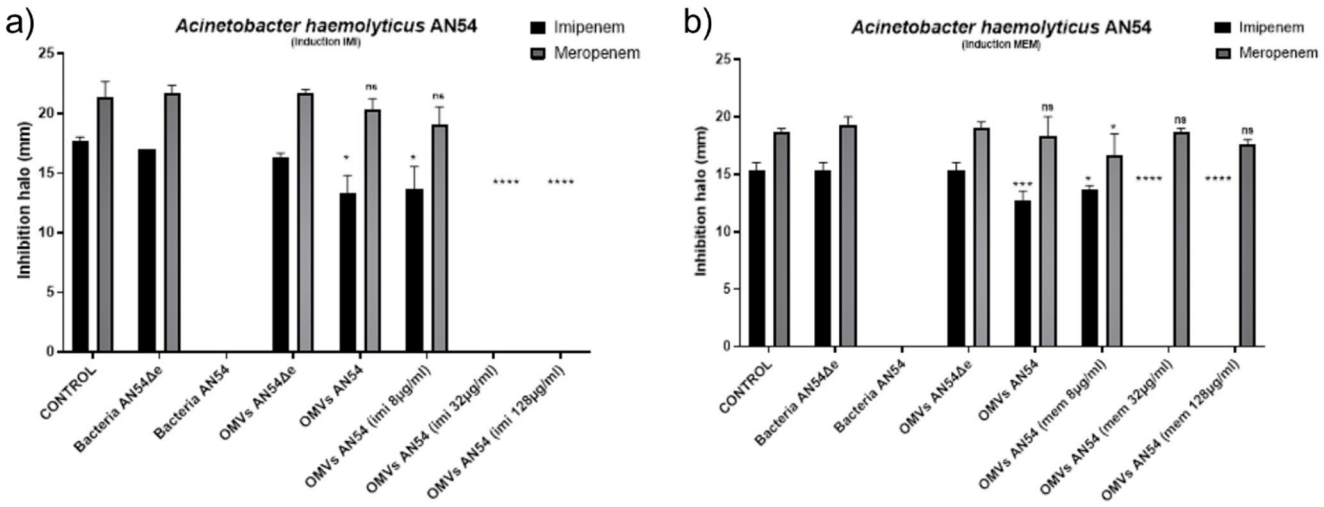

Fig. 2 The capacity of *Acinetobacter haemolyticus* AN54 OMVs to hydrolyze carbapenems. The inhibition halos obtained after performing the carbapenem inhibition test are shown. Purified AN54 OMVs were used in this assay after induction with carbapenems at different concentrations for OMV secretion (8, 32, and 128 µg/ml). AN54 **a** OMVs obtained after induction with imipenem, **b** induction with meropenem. *A. haemolyticus* AN54Δe was used as a negative control. The control of the test was a Sensi-Disk in BHI medium. The data correspond to three independent experiments and are shown as the mean value. Error bars indicate the standard deviation (SD) from triplicate measurements. Statistical analysis was performed using two-way ANOVA; ****p value < 0.0001

OMVs and OMVs from AN54Δe (control without NDM-1) showed considerably lower banding than those induced with carbapenems.

Furthermore, when comparing the protein profiles between replicates, the OMVs obtained after induction with imipenem were more stable and consistent, whereas those induced with meropenem showed greater variability. Based on these results, we selected OMVs obtained from AN54 after induction with 32 µg/ml of imipenem (AN54_Ind) due to their low variability between replicates and more defined banding pattern. Also, OMVs from AN54 and AN54Δe non-induced with antibiotics were selected to continue with high-complexity protein analysis by mass spectrometry to determine the protein content of these OMVs and evaluate the impact of the presence or absence of NDM-1 in the producing strain in the presence of imipenem.

Proteomic characterization of *Acinetobacter haemolyticus* OMVs

In the proteomic analysis of the OMVs from AN54Δe (control strain without bla_{NDM-1}), OMVs obtained from AN54 without antibiotic induction, and OMVs from AN54 with induction with 32 µg/ml of imipenem (AN54_Ind), a total of 760 proteins were identified, with 252, 268, and 240 proteins corresponding to the OMVs of AN54Δe, AN54, and AN54_ind, respectively. The proteomic comparison of the different OMVs (AN54Δe and AN54 or AN54_ind) is shown in Fig. 3.

We observed common proteins ranging from 126 to 142 between AN54Δe/ AN54_Ind and AN54Δe/AN54, respectively (Fig. 3a and b). On the other hand, AN54 and AN54_Ind share 173 proteins (Fig. 3c). It is important to highlight that among the three OMVs, 108 proteins are shared, which are mainly involved in metabolic pathways such as glycolysis, biosynthesis of amino acids, and proteins involved in gene expression, among others (Fig. 3d; Supplementary Table S1). The cellular localization of these proteins was primarily in the cytoplasm, outer membrane, and ribosomes. Also, 92, 61, and 49 proteins were exclusively present in the OMVs of AN54Δe, AN54, and AN54_ind, respectively. Although the proteins exclusive to each sample were related to common metabolic pathways, such as protein synthesis, amino acid metabolism, and general metabolism, they were not the same in each case (Supplementary Table S2).

On the other hand, concerning the mechanisms involved in the resistance, in the three OMV samples, porins like CarO, OmpA, OmpW, OprD, and efflux pumps such as AdeABC, and RND transporters were identified as part of the protein cargo but with different abundances (Fig. 3e). ADC (Ampc) was detected only in the OMVs of AN54Δe, while it was not identified in those of AN54 or AN54_Ind. In contrast, NDM-1 was found exclusively in the OMVs of AN54_Ind.

The OMVs confer protection to susceptible strains against imipenem

After confirming, by proteomics and hydrolytic activity, that OMVs obtained from AN54 induced with imipenem and meropenem carried active NDM, we evaluated their

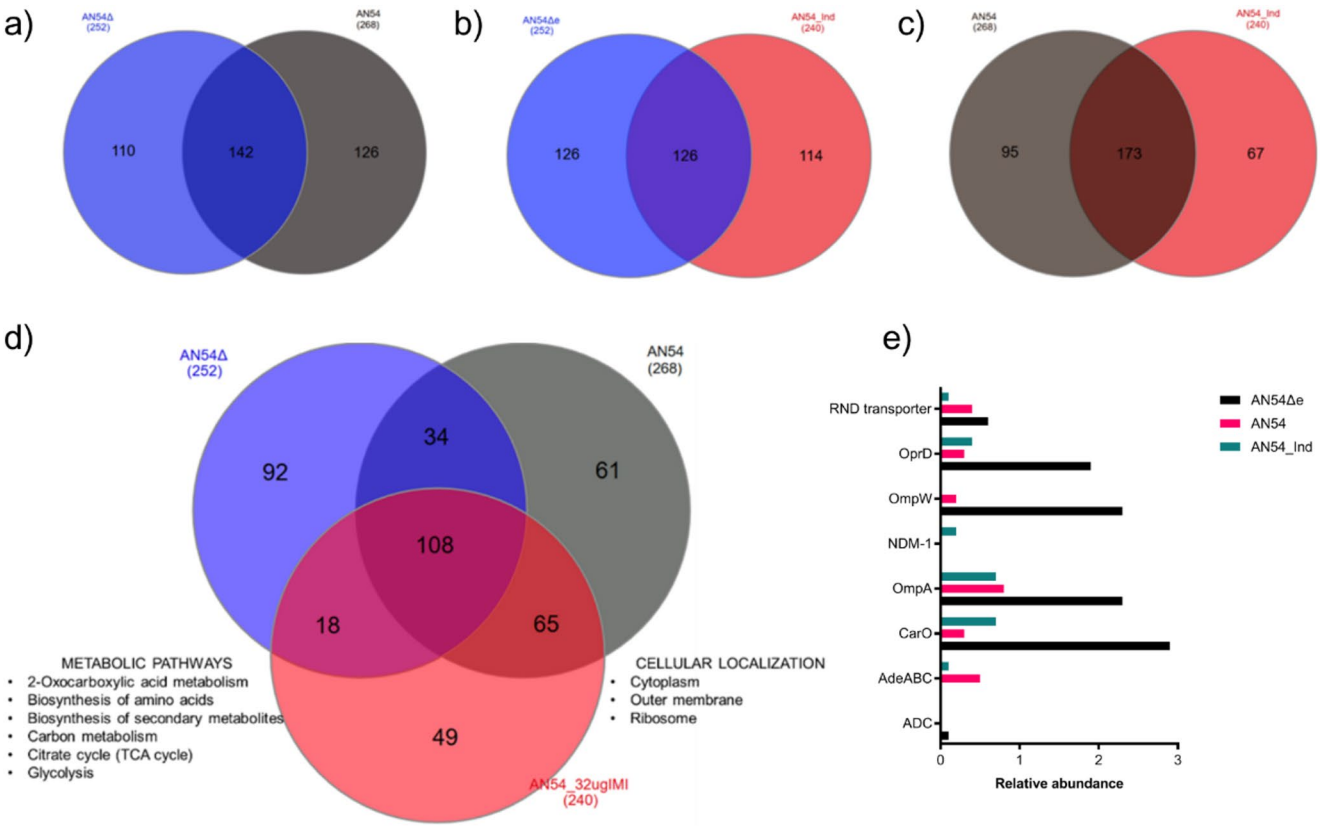

Fig. 3 Venn diagrams of proteins identified in OMVs. Comparative analysis of proteins obtained by mass spectrometry. **a**–**c** numbers of proteins common and unique to each group. **d** comparative analysis of the three OMV samples (AN54Δe, AN54, and AN54_Ind), including the main shared metabolic pathways and subcellular localization features, and **e** relative abundance of identified proteins involved in resistance identified in OMVs from AN54Δe (black bars), AN54 (pink bars), and AN54_Ind (green bars) are shown. AN54Δe was used as the control strain since it does not possess bla_{NDM-1}. The numbers indicate the identified protein counts. Proteomic profiling was performed once per sample

protective ability against carbapenems in susceptible strains, including *E. coli* ATCC 25,922, *A. baumannii* AE12, and AN54Δe. We observed that OMVs induced with carbapenems (8, 32, and 128 µg/ml) at concentrations of 0.5 µg/µl and 1.5 µg/µl of protein could protect all strains from imipenem (8 µg/ml), allowing growth during the first 24 h. However, for *E. coli* ATCC 25,922 and *A. baumannii* AE12, the susceptible phenotype was restored after the first subculture with the antibiotic. In contrast, with AN54Δe, antibiotic resistance persisted after successive passages in the presence of carbapenem, from which the transforming cells were recovered. These transformants were obtained after cocultures of AN54Δe with OMVs that had been previously induced with 8 µg/ml of meropenem (AN54Δe8MEM) and 128 µg/ml of imipenem (AN54Δe128IMI). This result suggested that bla_{NDM-1} was acquired via OMVs. To confirm this, transformants were recovered and tested using the CarbAcineto NP Test, which showed positive results for NDM-1 activity in both transformants (Fig. 4a). To determine whether this phenotype in AN54Δe8MEM and AN54Δe128IMI was due to the acquisition of the NDM-1 enzyme or if the OMVs had transferred the plasmid carrying the bla_{NDM-1}, the presence of the gene was investigated by PCR, revealing an amplified fragment of 813 bp (Fig. 4b) corresponding to the expected product. Based on these results, the detection of the pAhaeAN54e plasmid that carried bla_{NDM-1} in the strains AN54Δe8MEM and AN54Δe128IMI was carried out using PFGE (Fig. 4c), revealing the presence of a plasmid of approximately 45.46 Kbp in both strains, corresponding to the estimated size of plasmid pAhaeAN54e carried bla_{NDM-1} of the *A. haemolyticus* AN54 from which the OMVs were obtained.

Discussion

The spread of *Acinetobacter* species, especially *Acinetobacter baumannii*, has been associated with an alarming increase in hospital mortality rates, mainly affecting immunocompromised patients in intensive care units (ICUs) [38]. However, *A. baumannii* is not the only clinically relevant member of the genus. In recent years, several

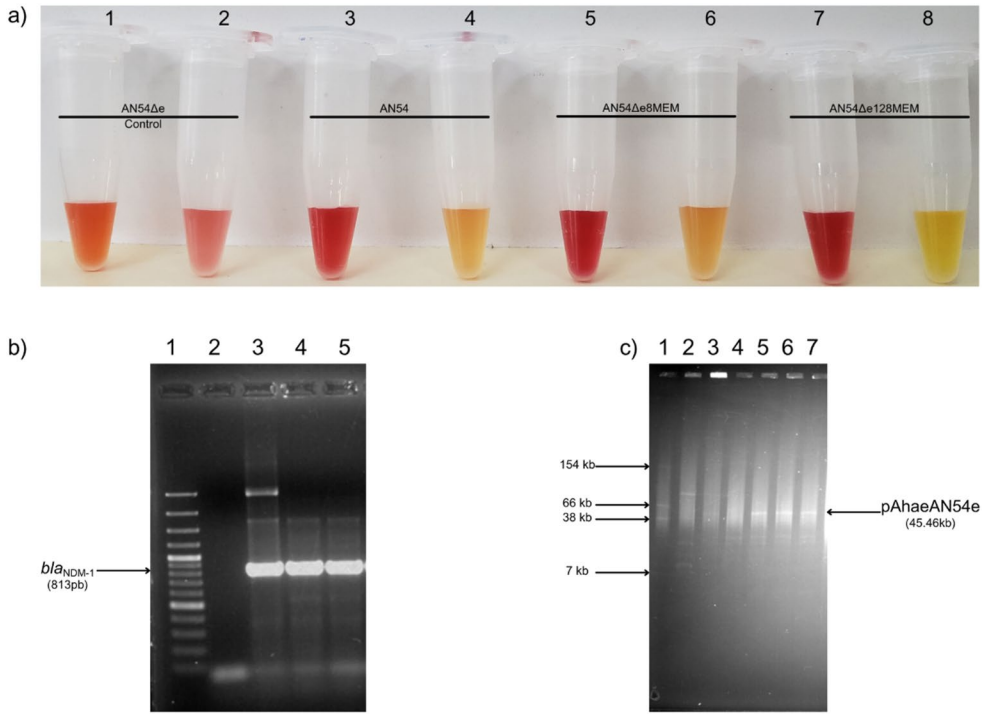

Fig. 4 Characterization of AN54Δe transformants. Molecular confirmation by CarbAcineto NP test, PCR, and PFGE of the acquisition of the resistance plasmid by the transforming strains. **a** Results of the CarbAcineto NP test with the transformants. Tubes are presented from left to right as indicated: 1, 3, 5 and 7 correspond to the negative controls (PF without imipenem) of the respective samples; 2, 4, 6 and 8 correspond to the positive control (PR with imipenem) of the respective samples. **b** Identification of bla_{NDM-1} by PCR. Lane 1, 100 pb DNA Ladder; Lane 2, AN54Δe; Lane 3, AN54; Lane 4, AN54Δe8MEM; Lane 5, AN54Δe128MEM. **c** PFGE-S1 gel. Line 1: *Escherichia coli* NCTC 50,192 (four plasmids of 154 kb, 66 kb, 38 kb, and 7 kb.); Line 2: AN11616; Line 3: AN54 (plasmid-bearing strain, pAhaeAN54e); Line 4: AN54Δe (plasmid-cured strain); Line 5–6: AN54Δe8MEM; Line 7: AN54Δe128MEM

non-*baumannii* species have gained medical importance due to their increasing ability to cause nosocomial infections. Among them are *Acinetobacter haemolyticus*, *A. lwoffii*, *A. ursingii*, *A. parvus*, and *A. junii*, which are part of the *Acinetobacter calcoaceticus-baumannii* complex [26].

The emergence of infections caused by these species has been favored by the presence of multiple antimicrobial resistance mechanisms in *Acinetobacter* spp. Carbapenems, considered first-line drugs due to their broad spectrum of activity and high efficacy against multidrug-resistant bacteria, have lost effectiveness as resistant strains have emerged [39]. This phenomenon is not limited to *A. baumannii* (CRAB) but has also been documented in *A. haemolyticus*, significantly complicating the treatment of infections caused by this pathogen [40]. In Mexico, the isolation of multiple clinical strains of *A. haemolyticus* has been reported, along with the presence of resistance genes such as bla_{NDM-1}, evidencing its emerging role as a potential reservoir and disseminator of antimicrobial resistance [26, 41].

Antibiotic resistance monitoring in the *Acinetobacter* genus has been biased by the almost exclusive reporting of *A. baumannii* strains due to its higher frequency of isolation, leading to the overlook of acquisition of relevant resistance genes, such as bla_{NDM-1}, in non-*baumannii* species like *A. haemolyticus* [26, 28]. This phenomenon affects epidemiology and clinical practice, complicating treatment decisions, especially now that new mechanisms of antibiotic resistance dissemination are being reported [42, 43]. Studies on releasing outer membrane vesicles (OMVs) have shown that resistance gene dissemination no longer relies solely on the previously studied conventional mechanisms [44]. In this work, we analyzed the role of OMVs in carbapenem-resistant *A. haemolyticus* under antibiotic-induced stress [44]. *A. haemolyticus* AN54 is a clinical strain that harbors resistance genes such as $bla_{OXA-265}$, *aac(6')-Ig*, *aphA6*, and bla_{NDM-1}, present in one of the five plasmids of the strain, as reported in a previous study [26].

The release of resistance genes via OMVs has been reported in *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and *A. baumannii*, where genes are transferred between bacteria of the same species and genus and different genera [2, 15–17]. However, the number of studies regarding active beta-lactamases released via OMVs has been limited.

It is important to note that the intrinsic protein characteristics of beta-lactamases are key to their release via OMVs [33]. NDM-1, a metallo-beta-lactamase with unique

properties, has rapidly spread since its first identification in 2009 [45]. Its anchoring to the membrane, mediated by its signal peptide and residues such as Cys26, Arg52, and Arg45, protects it from degradation by periplasmic proteases under low Zn (II) conditions and favors its packaging and release in OMVs as an active enzyme capable of contributing to bacterial resistance [18, 30, 33, 43].

In our study, we tested different conditions, incubation times, and carbapenem concentrations to obtain OMVs from our *A. haemolyticus* strain carrying NDM-1; because it has been reported that longer incubation times or severe stress result in a greater probability of obtaining E-type OMVs (cell lysis) rather than B-type ones (natural secretion) [46]. Under these conditions, transmission electron microscopy (TEM) demonstrated that AN54 releases B-type OMVs without altering membrane morphology, even under antibiotic stress. This stability may be attributed to NDM-1, which is anchored to the membrane and does not compromise bacterial fitness, as previously shown by López et al. [30]. These observations contrast with previous reports in *E. coli*, where peptidoglycan-level membrane alterations have been associated with OXA-type beta-lactamase expression, suggesting that envelope effects may differ depending on the type of beta-lactamase carried by each bacterium [30, 33, 47].

Additionally, we observed no differences in phenotype between OMVs released by the strain AN54 (with bla_{NDM-1}) and the control strain AN54Δe (without bla_{NDM-1}) by TEM. However, we observed an increase in OMVs numbers when the bacteria were exposed to antibiotic stress, suggesting that antibiotic pressure favors increased OMVs release, likely as a protective mechanism against the effects of the antibiotic on the secreting bacteria. These results coincide with those of Kesavan et al. [21], who observed increased release of OMVs after eravacycline induction in *A. baumannii* ATCC 19,606 and *A. baumannii* JU0126. Nevertheless, they observed no significant phenotypic differences between antibiotic-induced OMVs and OMVs obtained without induction. On the other hand, although the antibiotic resistance phenotype of the strains has not been linked to OMV morphology, OMVs from antibiotic-resistant strains have been observed to be more cytotoxic and immunogenic [24].

When assessing carbapenemase activity in OMVs released by AN54 under noninduced antibiotic conditions, carbapenemase activity was detected. These results suggest that, in the absence of antibiotic stress, released OMVs can degrade antibiotics in the environment. However, when OMV secretion was induced under antibiotic stress, they were able to completely degrade carbapenems in the medium. These findings could have important clinical implications for infections caused by carbapenemase-producing bacteria, as in the case of our strain AN54, which secretes OMVs harboring active NDM-1 carbapenemase even before the presence of antibiotics. Our results also suggest that OMVs can disseminate active carbapenemases, even in the absence of the antibiotic, thereby contributing to the spread of resistance among bacterial populations; however, further studies are needed to confirm this potential mechanism.

Carbapenemase activity mediated by OMVs has been previously reported in different models, such as *Stenotrophomonas maltophilia* (after imipenem, amoxicillin, and ticarcillin exposure), in *Klebsiella pneumoniae* (showing KPC and NDM-1 activity with meropenem induction), in *Escherichia coli* (showing NDM-1 activity with meropenem induction), and in *Acinetobacter baumannii* (showing OXA-24 and OXA-58 activity) [7, 16, 34, 43, 48]. This is the first study of an *A. haemolyticus* strain carrying NDM-1 in which OMV secretion was induced by two carbapenems at different concentrations, reveling greater activity against imipenem in the OMVs, attributable to NDM-1's affinity for this antibiotic.

To determine whether the presence of the bla_{NDM-1}-carrying plasmid and exposure to antibiotics alter vesicle contents, we analyzed the OMV protein profile obtained from AN54Δe and AN54 under different conditions. We observed variations in the protein profiles of the OMVs analyzed and identified common protein bands that were also conserved in the total bacterial extract, suggesting that some membrane proteins of the secretory strain are packaged into OMVs regardless of the stimulus. On the other hand, when analyzing the protein profiles of AN54 OMVs induced versus those of the AN54Δe control, we observe even greater variation. OMVs induced by imipenem exhibited a more defined profile, possibly due to more stable vesiculation and differences in protein synthesis. In contrast, induction with meropenem could elicit a more complex response, as reflected by greater differences in protein bands, although further studies are needed to corroborate this. However, our findings demonstrate that, despite belonging to the same antibiotic class, the two drugs differentially affect the protein profile of OMVs, underscoring the importance of performing proteomic analysis.

Proteomic analysis revealed that most of the proteins present in the OMVs come from the cytoplasm, outer membrane, and ribosomes, which is consistent with the findings of Dhurve et al. and Kesavan et al., who predominantly identified cytoplasmic proteins, followed by outer membrane and periplasmic proteins in OMVs derived from *A. baumannii* under antibiotic-free conditions and after exposure to eravacycline, respectively [21, 22].

These results are consistent with the theory proposed by Toyofuku et al., who suggested that type B OMVs may contain cytoplasmic proteins, a hypothesis supported by other

studies [4, 21, 22, 49, 50]. The nature of OMVs can explain their protein content, serving as reservoirs of bioactive metabolites and cellular waste, which are beneficial to the bacterial community. It has been suggested that factors such as the anchoring of proteins at their final location and the involvement of multifunctional or "moonlighting" proteins could influence their packaging [11, 51, 52]. However, the exact mechanism of protein selection remains unclear, and cytoplasmic content in OMVs is still not fully understood, as the incorporation of periplasmic and outer-membrane proteins is more expected given their biogenesis. In the OMVs analyzed in this study, we identified proteins associated with essential metabolic pathways, including glycolysis, the Krebs cycle, and amino acid biosynthesis, present in OMVs from AN54 under both uninduced and antibiotic-induced conditions. While the specific proteins within each pathway differed among OMV groups, they exhibited notable similarities. Some proteins, such as malate dehydrogenase and isocitrate dehydrogenase (involved in the Krebs cycle), glyceraldehyde-3-phosphate dehydrogenase, enolase, and phosphoglycerate kinase (involved in glycolysis), were common in AN54 OMVs, both with and without induction. In contrast, in AN54Δe, many proteins were outer membrane proteins (OMPs) and proteins associated with protein synthesis, such as ribosomal subunits.

Although, the role of OMPs in antibiotic resistance in *Acinetobacter baumannii* is well documented, their involvement in OMVs remains incompletely understood [49]. It has been suggested that they act as a gateway for the antibiotic into the vesicular lumen, where they can be degraded by beta-lactamases such as NDM-1. Kim et al. proposed this model after observing that antibiotic-resistant *E. coli* OMVs had a higher abundance of OmpC and OmpF [50]. When the genes encoding the corresponding porins were deleted, the antibiotic permeability of the OMVs secreted by the mutant strains decreased. In this context, the detection of OMPs such as CarO, OmpA, OmpW, OprD, and AdeABC, together with the RND transporter, across all OMV types analyzed in our study supports the idea that these components may contribute to antibiotic influx and/or efflux processes within OMVs, potentially facilitating subsequent degradation by enzymes such as NDM-1. Interestingly, we observed a higher abundance of these OMPs in the OMVs of AN54Δe compared to the OMVs of AN54, both with and without induction. This suggests that, following the loss of the plasmid carrying $bla_{\text{NDM–1}}$, AN54Δe mainly uses OMPs as a resistance mechanism, making them a major component of its OMVs protein cargo. In contrast, the lower abundance of OMPs in AN54 may be related to the energetic cost associated with the presence of the plasmid carrying $bla_{\text{NDM–1}}$. The acquisition and maintenance of a plasmid imply an energetic cost that can compromise cell viability. To ensure its persistence, mutual adaptation and coevolution occur in the bacterial hosts [51]. Xiang et al. [52] demonstrated that when *E. coli* acquired a plasmid carrying $bla_{\text{NDM–5}}$, it reduced the expression of outer membrane proteins while increasing the plasmid copy number. Therefore, it is likely that the lower abundance of OMPs in the OMVs of AN54 results from a metabolic adjustment to compensate for the energetic cost of replicating the resistance plasmid.

On the other hand, although plasmid replication may be prioritized over OMP production, we did not detect NDM-1 in OMVs from AN54 under noninduced conditions; however, it was present in OMVs obtained after induction. This may be because the NDM-1 concentration in the OMVs was below the detection limit of the MS/MS technique. Nonetheless, its presence was evidenced by carbapenemase activity in our phenotypic test. Prior induction with imipenem increased NDM-1 concentration in OMVs, allowing its detection by the MS/MS technique.

Besides, ADC β-lactamase was detected in OMVs from AN54Δe, confirming that cephalosporinases are also y transported by OMVs, even without induction. The presence of ADC, a chromosomal class C β-lactamase, in the OMVs of the control strain (which lost the resistance plasmid) and not in the OMVs of AN54 with or without induction can be explained by the prioritization of plasmid replication over other genes, such as chromosomal β-lactamases, resulting in lower ADC levels. The presence of ADC in OMVs has been previously reported by Kesavan et al. [21] in *A. baumannii*.

After confirming that OMVs released by AN54, both with and without induction, exhibed carbapenemase activity and that NDM-1 was present in one of them, we performed antibiotic protection assays using the OMVs on susceptible isolates. The results of this test indicate that the resistance phenotype in these strains is transient and depends on OMVs' carbapenemase activity rather than on genetic or protein transfer. Our results are consistent with those reported by Gonzales et al. [18], who used OMVs from *E. coli* harboring active NDM-1 in co-culture with a susceptible of *E. coli* strain and observed resistance to imipenem and cefotaxime. This phenotype was not maintained after subculture. On the other hand, AN54Δe was the only strain that was successfully transformed to acquire permanent resistance via uptake of the resistance plasmid, likely due to the close similarity between the genetic backgrounds of AN54 and AN54Δe.

Given that we established conditions for primarily recovering type B OMVs, we hypothesize that packaging the entire plasmid within the vesicular lumen after carbapenem induction may be attributable to plasmid over-replication, a strategy used by AN54 to manage immediate antibiotic stress. Further studies are required to confirm this hypothesis. On the other hand, we believe this result is not due to

the production of type E OMVs, since AN54's MICs for imipenem and meropenem are > 128 µg/ml, as previously reported by Bello-López et al. [26]. Our findings align with those of Rumbo et al. and Charteejee et al., who, using OMVs from various *A. baumannii* isolates, successfully transferred plasmids ranging from 11 kb to a megaplasmid of ~ 122 Kb to susceptible strains [2, 16].

Conclusions

This study presents, for the first time, the morphological characteristics of OMVs secreted by a clinical strain of *A. haemolyticus* carrying bla_{NDM-1}. These OMVs possess a single membrane (type B) and range in size from 10 to 50 nm. They transport the active enzyme NDM-1 along with other proteins involved in resistance, providing temporary protection to susceptible strains against carbapenems. In addition, they carry the bla_{NDM-1}-harbouring plasmid which can be transferred from OMVs to a strain lacks this plasmid. These findings highlight the importance of studying OMVs as potential targets for combating antimicrobial resistance.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical statement *Acinetobacter haemolyticus* AN54 isolate was collected during routine sampling, and patient data were anonymized. The protocol for this study was approved by the Ethical Committee of Hospital number: HNP/ENS/177/2016.

Competing interests The authors declare no competing interests.

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